Institute, we found that the CHUGA-F75 strain was sensitive to gentamicin (MIC = 0.125 mg/L), doxycycline (MIC = 1 mg/L), and ciprofloxacin (MIC = 0.016 mg/L) and resistant to sulfamethoxazole/trimethoprim (MIC = 32 mg/L).

F. marina was described as responsible for systemic disease in fishes (*Lutjanus guttatus*, the cultured spotted rose snapper) in Central America, whereas 4 *F. salimarina* strains have been isolated from costal seawater in Guangdong Province, China, and 1 strain of *F. salina* has been grown from brackish seawater and seaweed off the coast of Galveston, Texas, USA (*6*–*8*). To our knowledge, these *Francisella* spp. were not responsible for human infection so far. This report, like previous descriptions of human infections caused by emergent *Francisella* spp., highlights that environmental or fish-related *Francisella* spp. could be responsible for opportunistic human infections resembling tularemia.

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Surveillance of Rodent Pests for SARS-CoV-2 and Other Coronaviruses, Hong Kong

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We report surveillance conducted in 217 pestiferous rodents in Hong Kong for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). We did not detect SARS-CoV-2 RNA but identified 1 seropositive rodent, suggesting exposure to a virus antigenically similar to SARS-CoV-2. Potential exposure of urban rodents to SARS-CoV-2 cannot be ruled out.

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was first identified in Wuhan, China, in late 2019 (*1*) and soon spread globally. Although its zoonotic origin remains unclear, animal species potentially susceptible to reverse-zoonotic transmission from humans have been identified (e.g., cats, dogs, minks, deer), some of which (e.g., mink) might maintain the virus and pose a risk of future spillback to humans (*2*,*3*). Domestic animals and urban wildlife are of particular concern (*4*) because of their potential exposure to viruses shed within urban environments. Analysis of the angiotensin-converting enzyme 2 (ACE2) receptor across diverse vertebrates suggests a potentially wide breadth of SARS-CoV-2–susceptible mammal host species (*5*).

The rapid transmission and adaptation of SARS-CoV-2 in humans has been characterized by the evolution of variants of concern (VOCs). Several VOCs, particularly the Alpha (B.1.1.7), Beta (B.1.351), and Gamma (P.1) variants, have convergently evolved an amino acid residue change in the receptor binding domain of the spike protein (N501Y) that was also observed following serial passage of SARS-CoV-2 in BALB/c mice (*6*). Recent in vitro and in vivo experiments have demonstrated that these VOCs are capable of infecting laboratory rats and mice (*7*; Montagutelli X et al., unpub. data, https://doi.org/10.1101/2021.03.18.436013). Such evolutionary processes indicate a possible risk for reverse-zoonotic transmission of VOCs into urban rodents.

We hypothesized that locations with positive SARS-CoV-2 detection in sewage could also serve as key surveillance targets for potential exposure of pestiferous urban rodents to SARS-CoV-2 shed into the environment. We conducted sewage surveillance in Hong Kong to identify hidden infections and localized outbreaks of SARS-CoV-2 (*8*) during the fourth wave of COVID-19 in Hong Kong (Appendix, https://wwwnc.cdc.gov/EID/article/28/2/21-1586-App1.pdf).

During February 3–May 12, 2021, we sampled 217 rodents (*Rattus* spp.), 193 live-trapped rodents and 24 found dead near collection sites (Appendix Table 1). We collected 189 *R. norvegicus* and 28 *R. tanezumi* rats from 8 districts, the majority (n = 186) from Sham Shui Po, Yau Tsim Mong, and Kowloon City (Figure), where SARS-CoV-2 positive sewage has been reported.

We found samples from 1,702 swabs and tissues from 217 rats negative for SARS-CoV-2 by real-time quantitative PCR and 15 from 9 rats positive for murine alphacoronaviruses and betacoronaviruses using PCR and phylogenetic analysis (Appendix Table 2, Figure 1). Using ELISA, we identified 1 of 213 rodent serum samples from an *R. norvegicus* rat collected in Yau Ma Tei seropositive for SARS-CoV-2 (Table; Appendix Figure 2) and 11 samples inconclusive; only 1 of 2 replicates from 8 samples gave a positive absorbance result, and 1 or both replicates from 3 samples gave a borderline absorbance (Table; Appendix Figure 2). The unambiguously positive sample, from rat no. 213, was confirmed positive in surrogate virus neutralization testing (sVNT; 31.7% inhibition), but negative by plaque-reduction neutralization test ($PRNT_{90}$; <10 titers for 90% reduction). All 11 inconclusive samples were negative (<20% inhibition) by sVNT. As a pre–COVID-19 biological control to test for cross-sensitivity, 50 rodent serum samples collected in 2008 were examined by ELISA; none exhibited an unambiguously positive result.

Our rodent surveillance in Hong Kong revealed potential exposure to SARS-CoV-2, and although viral RNA was not detected, this could be a limitation of sample size if prevalence of active infection was low. One serum sample showed positive ELISA and sVNT results but negative $PRNT_{90}$ results. Previous research demonstrated that the sVNT used in our study has >98.8% specificity and sensitivity without cross-reaction to alphacoronaviruses and murine betacoronavirus (*9*). Some sVNT-positive COVID-19–confirmed patients did not meet the threshold for positivity by $PRNT_{90}$ (*9*). This finding suggests that the seropositive result for SARS-CoV-2 or a closely related virus in the brown rat was unlikely to be attributable to past exposure to murine alphacorona-

viruses or betacoronaviruses.

During our study period, SARS-CoV-2 infection was reported in several imported and local human cases in multiple locations and in multiple sewage results. Before December 2020, SARS-CoV-2 locally circulating in Hong Kong predominantly carried 501N with presumably lower rodent infectivity; however, during our study period, Hong Kong reported many imported cases of SARS-CoV-2 variants, including B.1.1.7 and B.1.351, carrying 501Y, which has been demonstrated in mouse experiments to be a critical genetic adaptation (*6*). These imported cases might disseminate virus into the environment near quarantine hotels, presenting an increased risk of spillover into urban rodent populations and requiring enhanced biosecurity to

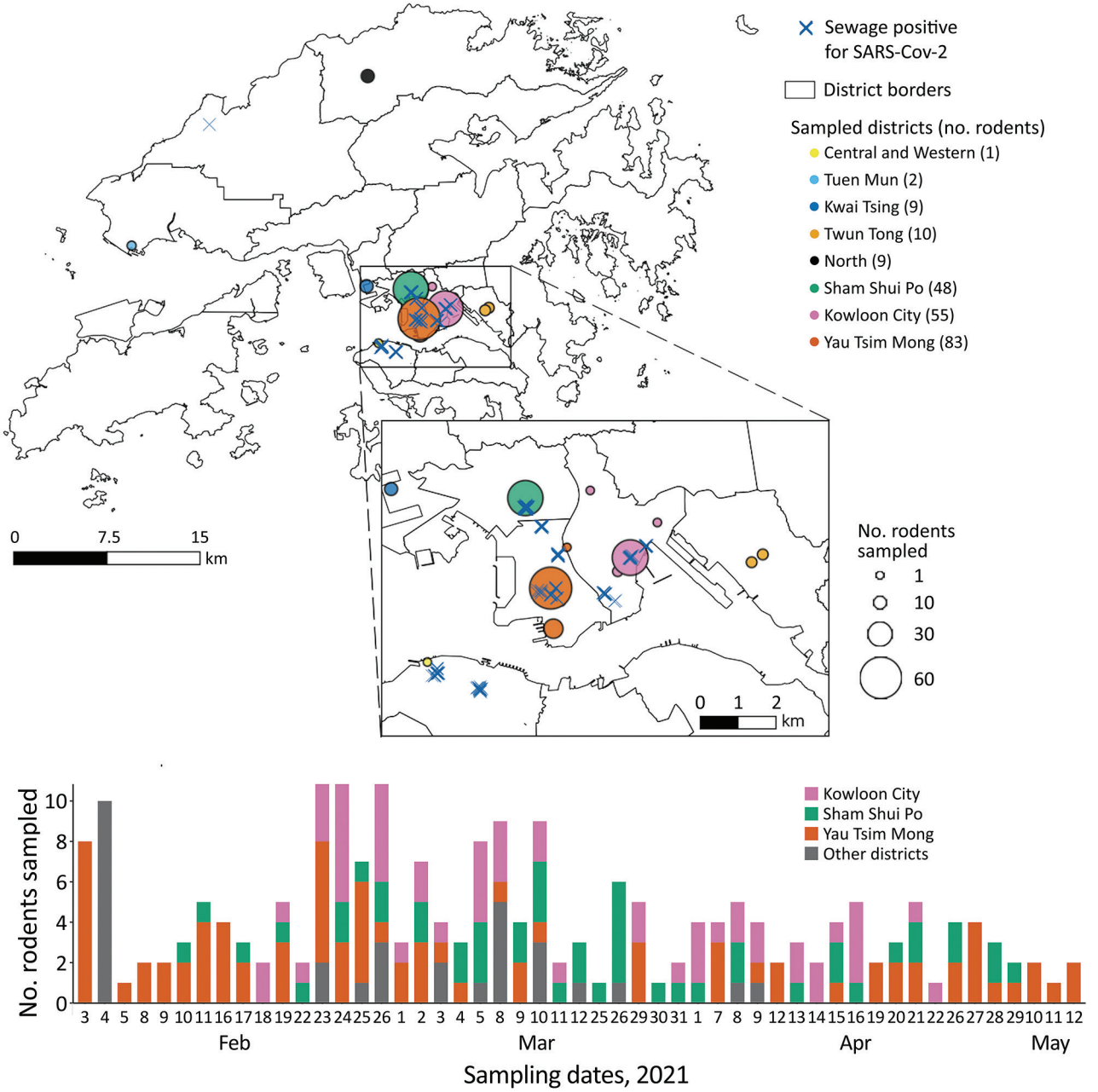

Figure. Surveillance of rodents for SARS-COV-2 conducted February–May 2021 in Hong Kong. A) Sampling sites, with number of rodents sampled and sewage testing positive for SARS-COV-2. Each circle represents a sampling location, color-coded by district and sized proportional to the number of captured rodents. Blue crosses represent locations where sewage was reported positive for SARS-COV-2during January 19–March 30, 2021. B) Number of sampled rodents, by collection dates and district. SARS-COV-2, severe acute respiratory syndrome coronavirus 2

Table. Information on rodents with unambiguous (n = 1) or inconclusive (n = 11) positive serum samples in ELISA testing in study of surveillance of rodent pests for severe acute respiratory syndrome coronavirus 2 and other coronaviruses, Hong Kong*

Animal code	*Rattus* species	Collection date	District	ELISA A/CO		sVNT, inhibition, %
				1st replicate	2nd replicate	
Rat-027	*R. tanezumi*	Feb 11	Sham Shui Po	0.019	0.855	1.281
Rat-069	*R. norvegicus*	Feb 24	Kowloon City	0.837	0.964	0.991
Rat-070	*R. norvegicus*	Feb 24	Kowloon City	1.199	0.472	–2.128
Rat-073	*R. tanezumi*	Feb 25	Yau Tsim Mong	1.445	0.033	2.224
Rat-076	*R. norvegicus*	Feb 25	Sham Shui Po	1.644	0.027	1.136
Rat-089	*R. norvegicus*	Mar 1	Yau Tsim Mong	1.324	–0.041	1.209
Rat-090	*R. norvegicus*	Mar 1	Yau Tsim Mong	1.636	–0.027	–0.532
Rat-096	*R. norvegicus*	Mar 2	Yau Tsim Mong	0.934	–0.007	3.748
Rat-097	*R. norvegicus*	Mar 2	Yau Tsim Mong	1.592	0.013	–4.666
Rat-098	*R. tanezumi*	Mar 2	Sham Shui Po	1.920	–0.724	–2.466
Rat-102	*R. norvegicus*	Mar 3	Kwai Tsing	0.992	–0.499	0.145
Rat-213†	*R. norvegicus*	May 10	Yau Tsim Mong	13.643	14.497	31.7

*A/CO was interpreted as negative if <0.9, borderline if 0.9–1.1, and seropositive if >1.1, according to manufacturer instructions. Serum was considered unambiguously positive if both replicates were seropositive. Positive cutoff for sVNT was 20% inhibition, as described elsewhere (*9*).
A/CO, absorbance cutoff; sVNT, surrogate virus neutralization test.
†Positive in both ELISA and sVNT.

limit potential exposure to urban rodents or other susceptible animals. Our finding of potential SARS-CoV-2 exposure in a pestiferous rat highlights the need for sustained monitoring of rodent populations to rapidly detect spillover events and subsequently put in place timely interventions (e.g., disinfestation using trapping and pesticide) to prevent potential establishment of new reservoirs.

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Surveillance of Rodent Pests for SARS-CoV-2 and Other Coronaviruses in Hong Kong

Appendix

Materials and Methods

Sample Collection

Rodents from the genus *Rattus* (determined by morphology and confirmed using DNA barcoding) were trapped and sampled as part of rodent surveillance conducted during February–May 2021, in collaboration with the Food and Environmental Hygiene Department (FEHD) of Hong Kong SAR. Additional traps were placed in back alleys close to known SARS-CoV-2–positive sewage sites in Sham Shui Po, Yau Tsim Mong, and Kowloon City districts (Figure). Live-trapped rodents (n = 193) were euthanized with an overdose of isoflurane. Samples for pathogen surveillance were collected post-mortem. Rodents found dead near the sampling sites were also collected (n = 24).

Blood was collected by cardiac puncture and swab samples including body surface, oropharyngeal, and rectal swabs were collected in duplicate for all subjects. For rodents captured alive, a full necropsy was performed to collect most of the major organs (i.e., lymph nodes, heart, lung, trachea, liver, spleen, small and large intestine, kidney, bladder, and brain). When available, urine, feces, ectoparasites, and endoparasites were also collected. For dead rodents, except for 4 specimens, blood was collected in the thoracic cavity as well as the whole heart after a partial necropsy.

Blood samples were collected in CAT serum clot activator coated tubes to retrieve serum. In the case of dead rats, when possible, 1mL of phosphate buffer saline 1X was added to the blood and heart in CAT serum activator coated tube. Swab samples were collected in virus transport media (VTM) containing M199 media, antimicrobials, antifungal, bovine serum albumin and stabilizers previously described (*1*). Tissue samples were collected using standard

sterile techniques in microbiological practice and stored in VTM as well as in RLT lysis buffer (QIAGEN). Samples were transported on ice for same-day processing or dry ice to the State Key Laboratory of Emerging Infectious Diseases at the University of Hong Kong where further sample processing was conducted. The research protocol was approved by the University of Hong Kong Committee on the Use of Live Animals in Teaching and Research (CULATR 5657–21).

RNA Extraction

RNA was extracted from swab (n = 651), urine (n = 94), and blood samples (n = 194, the first 23 were not extracted) using QIAamp Viral RNA Mini Kit (QIAGEN, https://www.qiagen.com), and from tissue samples (i.e., lymph nodes, lung, trachea, small intestines, n = 558) using RNeasy Plus Micro Kit (QIAGEN). Both kits were used according to manufacturer instructions except for the elution step. Elution was performed twice with 30 μL buffer AVE for QIAamp or RNase-free water for RNeasy with 5 min incubation at room temperature each time.

SARS-CoV-2 Detection

The COVID-19 Real-Time PCR Kit (Chaozhou Hybribio Biochemistry Ltd, http://hybribio.com) was used for quantitative reverse transcription PCR with multiple fluorescence detection channels including FAM targeting SARS-CoV-2 ORF1ab, HEX targeting SARS-CoV-2 N region, and Cy5 targeting B2M gene as an internal control. We confirmed that this method was able to detect representative human SARS-CoV-2 circulating in Hong Kong during our sampling (WHP-4212) as well as from 1 imported case harboring the N501Y mutation (WHP-4238), which yielded a cycle threshold value <30.

Universal Coronavirus Detection

The presence of other coronaviruses was assessed by a 2-step RT-PCR reaction to generate a 442 bp amplicon using universal coronavirus primers (UniCoV) targeting the most conserved region of the RNA-dependent RNA polymerase gene (CorUniF: 5′-ATGGGTTGGGATTATCCTAAGTGTGA-3′, CorUniR2: 5′-CATCATCAGATAGAATCATCATAG-3′, and CorUniR3: 5′-CCATCATCAGATAGAATCATCAT-3′ (*1*). Total RNA was reverse transcribed into complementary DNA (cDNA) using PrimeScript RT Master Mix (TaKaRa,

https://www.takarabio.com) using the following program: 15 min at 37°C followed by 5 sec at 85°C. The 20-µL reaction volume contained 4 µL of 5X PrimeScript RT Master Mix (Perfect Real Time) and 16 µL of RNA extract. The cDNA was subsequently amplified using AmpliTaq Gold DNA polymerase (ThermoFisher Scientific, https://www.thermofisher.com). The 25-µL reaction volume contained 2.5 µL 10x PCR Gold buffer, 1.5 µL $MgCl_2$ (25 mmol), 0.5 µL dNTP mix (10 mmol), 0.5 µL of forward primer CorUniF, 0.25 µL of reverse primers CorUniR2 and CorUniR3, 0.25 µL AmpliTaq Gold DNA polymerase (5 U/µL), and 1 µL of cDNA template. Amplification was performed using the following program: 5 min at 95°C, 45 cycles of 30 sec at 95°C, 30 sec at 48°C, and 45 sec at 72°C, followed by a final extension step of 5 min at 72°C. Amplicons were visualized by electrophoresis on a 1.5% agarose gel. PCR positive samples were purified using Expin PCR SV kit (GeneAll, https://www.pcr-lab-products.com) following manufacturer instructions and sequenced using an ABI 3730xl DNA Analyzer at the Centre for PanorOmic Sciences (http://www.med.hku.hk/en/research/facilities-and-services/cpos) (CPOS) to confirm the presence and identity of coronaviruses using BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nucleotide database in GenBank. The amplicon sequences were submitted to GenBank under accession numbers OK018140 – OK018153.

DNA Extraction and Rodent Species Identification

DNA was extracted from the ear tissue of each animal using the DNeasy Blood & Tissue Kit (QIAGEN). Conventional PCR was used to amplify a 708 bp region of the mitochondrial cytochrome c oxidase subunit I (COI) gene (Rat-COI-F: 5′-CGTTGACTMTTTTCAACYAACCAC-3′, Rat-COI-R 5′-CRTGTGARATAATTCCAAAYCCTGG-3′) to confirm the species of each animal. If DNA extracted from ear tissue failed to yield an amplicon, cDNA from swabs or tissue samples were used as input for PCR. Barcoding of the COI region was performed using AmpliTaq Gold DNA polymerase (ThermoFisher Scientific) in a reaction volume of 25 µL containing 2.5 µL 10x PCR Gold buffer, 1.5 µL $MgCl_2$ (25 mmol), 0.5 µL dNTP mix (10 mmol), 0.5 µL of forward primer Rat-COI-F, 0.5 µL of reverse primer Rat-COI-R, 0.25 µL AmpliTaq Gold DNA polymerase (5 U/µL), and 2 µL of DNA or cDNA template. The barcoding PCR was performed using the following thermocycling program: 5 min at 94°C, 40 cycles of 30 sec at 94°C, 40 sec at 54°C, and 60 sec at 72°C followed by a final extension step of 10 min at 72°C. Amplicons were

visualized by electrophoresis on a 1.5% agarose gel. Amplicons were purified using Expin PCR SV kit (GeneAll) following manufacturer instructions and sequenced using an ABI 3730xl DNA Analyzer (ThermoFisher Scientific) at CPOS.

Phylogenetic Analysis

The gene sequences RNA-dependent-RNA-polymerase of the coronaviruses identified in our rodent samples were aligned with selected previously published coronavirus sequences (largely alphacoronavirus and betacoronavirus) using MAFFT v7.273 (*2*). The multiple alignment was manually checked for accuracy and poor gap regions were trimmed. A phylogenetic tree was estimated using the maximum likelihood method and GTRGAMMA substitution model implemented in RAxML v8.2.12 (*3*). One hundred multiple inferences were performed, and the best tree was selected for comparison with 500 bootstrap replicates.

Serology

Detection of antibodies with activity against the SARS-CoV-2 spike protein was performed on heat-inactivated serum using the WANTAI SARS-CoV-2 Ab ELISA Diagnostic Kit (Beijing Wantai Biologic Pharmacy Enterprise Co., Ltd, https://www.ystwt.cn), a double-antigen binding assay for detection of total antibodies to SARS-CoV-2, following manufacturer instructions. Absorbance was measured at 450nm with a reference wavelength set at 620 nm using a FilterMax F5 multimode microplate reader. Each sample was tested twice following manufacturer recommendations and established cutoff values for positive (absorbance/cutoff value >1.1) or borderline samples (absorbance/cutoff value 0.9–1.1). Test results were considered valid if the absorbance of the 2 internal positive controls were ≥0.19 and if the absorbance of the 3 internal negative controls were ≤0.1. For each test, cutoff values were calculated as mean absorbance of the 3 internal negative controls (use 0.03 if <0.03) plus 0.16. In the case of unambiguous positive (i.e., both replicates showing absorbance/cutoff values >1.1) and inconclusive (i.e., only 1 of the 2 replicates giving a positive absorbance/cutoff ratio >1.1, or with 1 or both replicates giving a borderline absorbance/cutoff ratio of 0.9–1.1) results from ELISA, the samples were further tested using an in-house SARS-CoV-2 surrogate virus neutralization test (sVNT) as described elsewhere (*4*,*5*). This method has been validated not to cross-react with serum of rodents containing antibodies against murine hepatitis virus as well as serum containing antibodies to several other epizootic alpha- and betacoronaviruses (*4*). An in-

house plaque-reduction neutralization test described elsewhere (*4*) was used to further investigate the sVNT-positive finding.

Fifty rodent serum samples collected in 2008 were examined by ELISA as a pre-COVID-19 biologic control. Their resulting absorbance/cutoff values were between −0.096 to 2.070. Two of the pre-COVID-19 serum samples showed inconclusive results in the ELISA; none exhibited unambiguously positive results.

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Appendix Table 1. The number of rodents (*Rattus* spp.) sampled from urban areas within Hong Kong

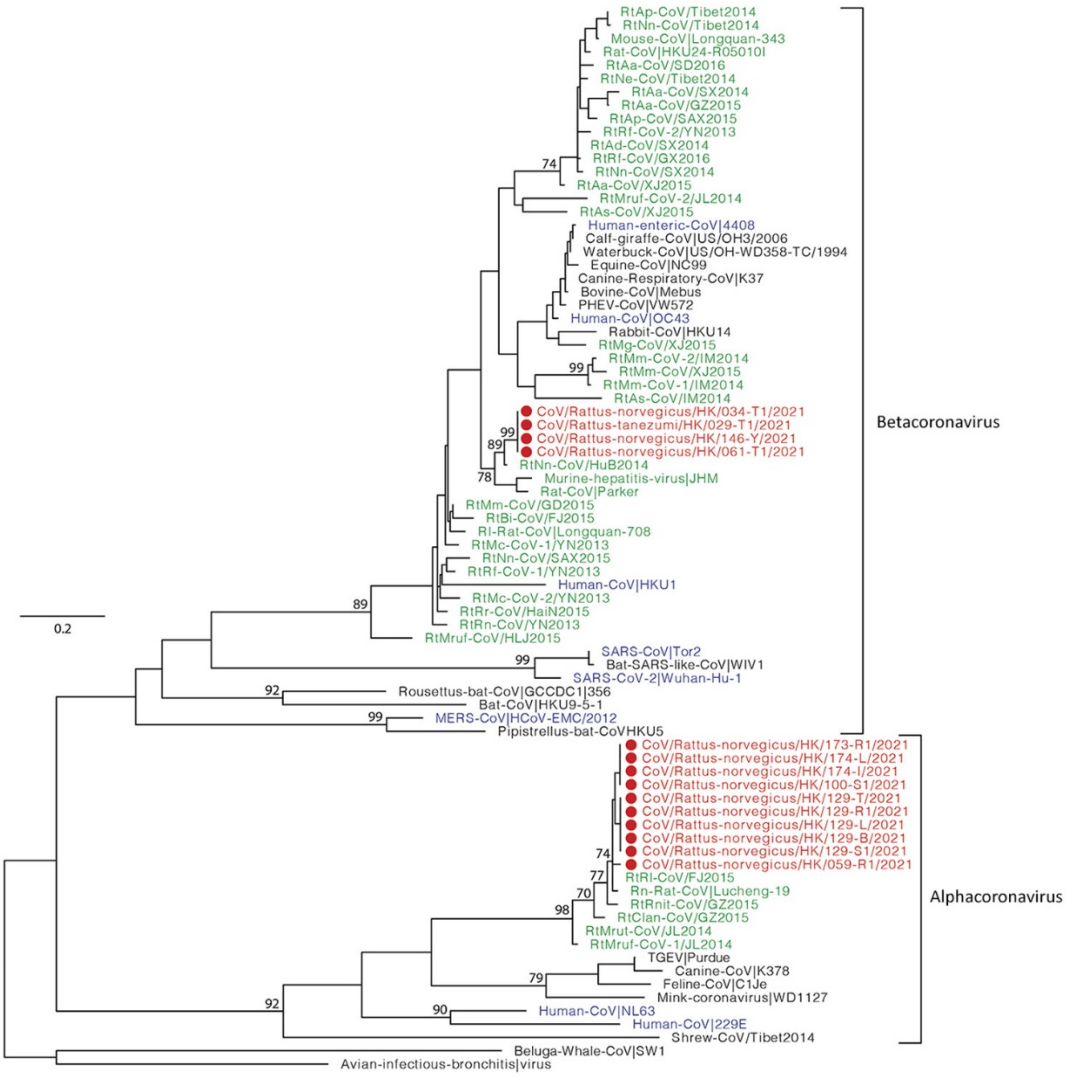
District	Locality	No., *R. norvegicus* sampled alive; dead	No., *R. tanezumi* sampled alive; dead
Hong Kong Island			
Central and Western	Sai Wan	0, 0	1, 0
Kowloon			
Kowloon City	Hung Hom	4, 0	0, 0
	Kai Tak	1, 0	0, 0
	Kowloon city	0, 0	1, 0
	To Kwa Wan	46, 6	3, 0
Kwun Tong	Kwun Tong	4, 0	1, 0
	Ngau Tau Kok	4, 0	1, 0
Sham Shui Po	Sham Shui Po	46, 2	2, 0
Yau Tsim Mong	Ho Man Tin	1, 0	0, 0
	Jordan	1, 0	0, 0
	Tsim Sha Tsui	15, 3	5
	Yau Ma Tei	*47, 9	13, 4
	Unknown	0, 0	1, 0
New territories			
Kwai Tsing	Kwai Chung	9, 0	0, 0
North	Sheung Shui	9, 0	0, 0
Tuen Mun	Tuen Mun	2, 0	0, 0
Total		189, 20	28, 4

*Single seropositive rat, Rat-213, captured in this location.

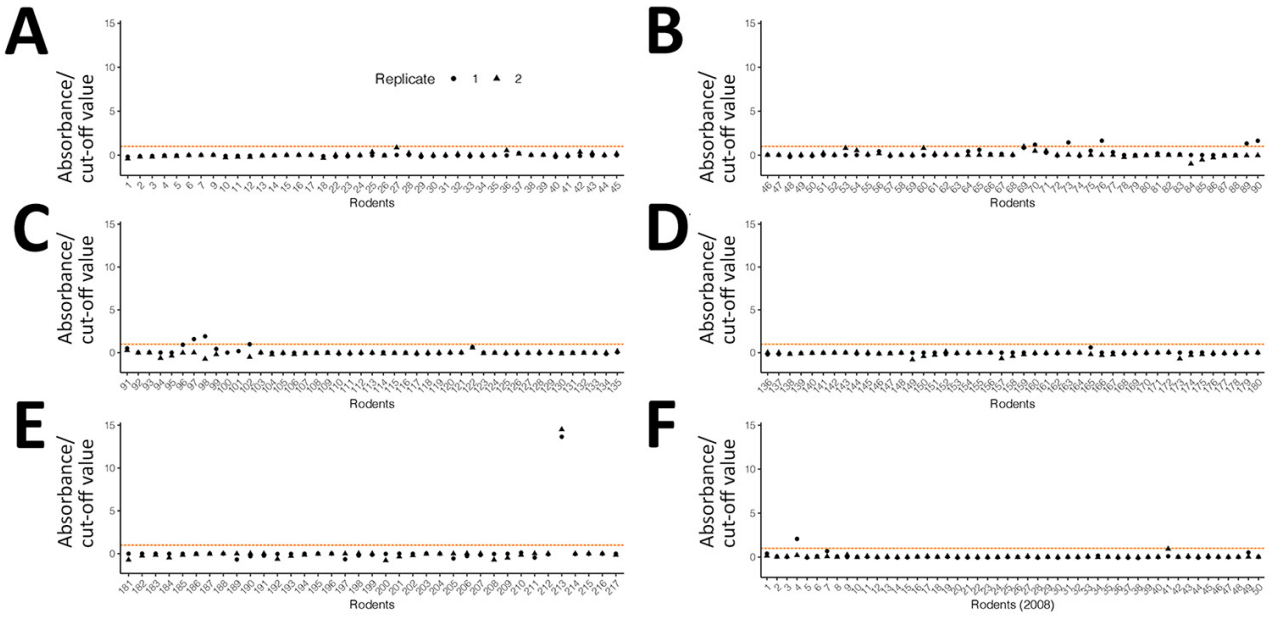
Appendix Table 2. PCR detection of SARS-CoV-2 and other coronaviruses in rodents from Hong Kong*

Sample types	SARS-CoV-2 positive	UniCoV positive
Body surface swab	0/217	2/217
Oropharyngeal swab	0/217	3/217
Rectal swab	0/217	3/217
Blood	0/194	1/194
Lymph node	0/186	1/186
Lung	0/186	2/186
Trachea	0/186	2/186
Small intestine	0/186	1/186
Urine	0/94	0/94

*SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; UniCoV, universal coronavirus.



Appendix Figure 1. Phylogenetic tree showing the evolutionary relationship of alphacoronavirus and betacoronavirus found in the rodent samples collected February–May 2021. The tree was estimated based on the universal coronavirus primers amplicon region (located within the RNA-dependent-RNA-polymerase) using a maximum likelihood method. Rodent samples reported in this study are indicated with dots and in red (i.e., 14 specimens from 9 individual rodents; details in Appendix Table 2; Rat-034 tracheal sample was omitted from the tree due to poor sequencing quality). Reference coronavirus sequences from humans are indicated in blue and from rodents in green. Bootstrap support values (percentage; from 500 bootstrap replicates) for selected lineages are shown.



Appendix Figure 2. ELISA of the 213 rodent serum samples collected in 2021 (A, B, C, D, and E) and the 50 rodent serum samples collected in 2008 that we used as pre-COVID-19 biologic controls (F). Absorbance/cutoff value is interpreted as negative if <0.9, borderline if 0.9–1.1, and seropositive if >1.1. Each serum sample was tested twice, and the rodent considered unambiguously positive if both replicates were seropositive. The red dashed line represents the seropositivity threshold (absorbance/cutoff: >1.1) and the orange-shaded area represents borderline samples (absorbance/cutoff: 0.9–1.1).